A Rapid, Quantitative Procedure for Measuring the Unsaponifiable Matter from Animal, Marine, and Plant Oils[1]

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ABSTRACT

A simple, rapid, quantitative procedure for measuring the unsaponifiable matter (USM) in animal, marine and plant oils is described. Saponification is achieved by grinding the oil with potassium hydroxide pellets followed by a short heating period. The resultant soap mixture is then ground together with Celite powder, transferred to a glass column, and the USM is eluted with small amounts of dichloromethane. Good agreement between duplicate determinations was obtained for all oils examined. Values for the USM of 21 different fats and oils are given. Comparison of some of these values was made with those obtained with Official Methods.

INTRODUCTION

The amount of unsaponifiable matter (USM) found in animal, marine and plant oils is normally measured by modifications of a technique first developed in the nineteenth century (1). These procedures generally involve the saponification of the sample in methanolic potassium hydroxide as the first step in the analysis followed by recovery of the USM from the aqueous alcoholic medium. Methods used to recover the USM from the saponification media have been diversified; both the choice of solvents employed and the efficiency of extraction used have been reported to influence widely the final results obtained (2). The Official AOCS Method Ca6a-40 (3) requires the use of petroleum ether as the extractant, but reports indicate that not all polar constituents of the USM are entirely removed from the aqueous phase by this solvent (2). Other problems encountered in the conventional saponification technique include: difficulties in obtaining agreement between duplicate analysis (2); contamination of the USM fraction by soap and other residues and procedural problems in carrying out the actual analysis (2,4).

Recently this laboratory had to assess the amounts of certain constituents present in the USM of tallow. It was necessary to develop a procedure for the rapid saponification of oils which obviated the problems encountered with most existing methods as described above, and we have reexamined a procedure developed previously in this laboratory for the "dry" saponification of butteroil (5) for use with oils from other sources. In this method, no solvent was employed during saponification, and only small amounts of benzene were required for quantitative elution of USM from the resultant soap mixture (5). As the method was facile, we have examined it further, modified it where necessary and expanded its scope to include most common animal, marine and plant oils.

EXPERIMENTAL

Materials

Porcelain mortar 400 ml (Coors No. 14) capacity; glass pestle 15 cm or longer with smooth sides and no lip; glass column, 30 mm i.d. and 25 to 30 cm overall length with drip tip 5 cm x 8 mm o.d.; tamping rod; teaspoon, with long handle, stainless steel; potassium hydroxide pellets, ACS grade, J.T. Baker Chemical Co., Phillipsburg, NJ; calcium chloride pellets, J.T. Baker Chemical Co.; Celite 545 (Grade: not acid washed C-212), Fisher Scientific Co., King of Prussia, PA; dichloromethane, ACS grade, J.T. Baker Chemical Co. The dichloromethane was residue free.

Preparation of Calcium Chloride/Celite Mixture

A convenient amount of this mixture is prepared by grinding in a large mortar until uniform, one part 50% calcium chloride solution with three parts of Celite 545. The mixture is stored covered.

General Saponification Procedure

The complete saponification step is carried out in the 400 ml mortar. Potassium hydroxide pellets (3 g) are placed in the mortar, and distilled water (1 ml) is added dropwise to wet the pellets. The oil (ca. 5 g) is transferred from a weighed vessel to cover the pellets, and the exact weight of the oil taken is recorded by difference. The oil-alkali mixture is ground with a pestle until the pellets disintegrate and the mixture becomes smooth and viscous from soap formation. The grinding procedure is finished in less than 5 min, and saponification is brought to completion by heating the mortar and pestle in a 100 C oven for 20 min.

Extraction of Nonmarine Oils

After heating, the mortar is removed from the oven and cooled. Using the teaspoon, the soap is carefully scraped from the side of the mortar and from the pestle and reground until the mixture is granular. Approximately 15 g of Celite 545 is added in small batches to the contents of the mortar, with continuous grinding, and the mixture is reground until all of the soap is adsorbed and all of the material is powdered. The glass column is fitted with a plug of glass wool in its tip and charged with 5 g of the $CaCl_2$/Celite mixture. This powder is lightly tamped, and the contents of the mortar are transferred quantitatively to the column with the aid of the spoon. To recover residual traces of soap from the mortar, the mortar, pestle, tamping rod, and spoon are rinsed with 10-20 ml dichloromethane, and the rinsings are pipetted onto the column along the walls. After the rinsings have percolated into the packed bed, sufficient dichloromethane to wet the entire bed is added slowly, and the column is charged with solvent to a volume of 120 ml. The first 100 ml is collected in a graduated beaker. This volume of solvent (100 ml) is generally sufficient to extract completely the USM, but another 20 ml of solvent is added to the column to check on the completeness of extraction. This 20 ml effluent is collected, evaporated to dryness and examined for residue. This procedure is repeated if any residue is observed.

The solvent is removed under a stream of nitrogen with gentle heating. Residues in the beakers are taken up in small amounts of dichloromethane and transferred to a common beaker. Remaining solvent is removed under nitrogen and taken finally to constant weight under vacuum. Alternatively, the procedure may be shortened by placing a tared collection beaker on a hot plate in a hood simultaneously collecting the effluent and evaporating it under a stream of nitrogen.

[1]Presented at the AOCS Meeting, St. Louis, May 1978.

TABLE I

Unsaponifiable Content of Representative Fats and Oils Determined by the "Dry" Method

Oil	% USM		Oil	% USM	
Animal			Peanut (refined) B	0.40	0.40
			Rapeseed (unrefined) A	1.39	1.39
Beef oil[a]	0.24	0.24	Rapeseed (unrefined) B	1.35	1.38
Beef tallow A	0.20	0.21	Rapeseed (refined) A	1.30	1.34
Beef tallow B	0.73	0.74	Rapeseed (refined) B	1.48	1.50
Beef tallow C	0.57	0.59	Olive A	0.86	0.88
Butterfat	0.33	0.36	Olive B	0.80	0.84
Lard A	0.16	0.17	Safflower	0.61	0.62
Lard B	0.32	0.36	Soy (refined)	0.70	0.78
			Soy (unrefined)	0.85	0.86
Plant			Soy (unrefined A	1.02	1.02
			Soy (unrefined) B	1.09	1.12
Castor[b]	0.32	0.32	Tall	3.07	3.16
Cocoa butter	0.46	0.47			
Coconut (unrefined)	0.22	0.22	Marine		
Corn	1.52	1.54			
Corn (unrefined) A	2.03	2.07	Dogfish (refined)	15.42	15.73
Corn (unrefined) B	2.10	2.40	Herring (refined)	0.69	0.76
Cottonseed	0.76	0.77	Herring (unrefined)	1.10	1.20
Cottonseed (unrefined)	0.82	0.86	Redfish (refined)	0.66	0.69
Linseed (raw)	1.37	1.40	Redfish (unrefined)	1.15	1.17
Linseed (refined)	1.26	1.29	Sperm whale	34.17	34.18
Palm	0.19	0.21			
Peanut (unrefined) A	0.38	0.38			

[a]Oil isolated from the fractionation of tallow, F.W. Luddy, J.W. Hampson, S.F. Herb, and H.L. Rothbart, JAOCS 50:240 (1973).

[b]1.5 ml water required in mixing process to effect complete saponification.

Extraction of Marine Oils

Fish oils are handled as described above except for the following modifications: (a) saponification is brought to completion by heating the mortar and pestle in an oven set at 125-130° for 30 min, and (b) the column is charged instead with about 200 ml dichloromethane. The first 150 ml of solvent is collected in a graduated beaker and subsequent fractions are collected in 50 ml volumes. Elution from the column is continued until no residue remains in the last beaker after removal of the solvent. After the column is stripped of the USM, collected fractions are combined and the residue is weighed as described previously.

Examination of USM for Unsaponified Glycerides, Soaps, and Other Artifacts

Triglycerides. Each USM residue (Table I) was diluted to 1 ml with dichloromethane. Aliquots (~200 μg of USM) were spotted on a TLC plate (Silica Gel G, 500 micron, Analtech, Inc., Newark, DE) along with the following three triglyceride standards: glyceryl-*rac*-1,2-dipalmitate-3-oleate; glyceryl-*rac*-1,2-distearate-3-palmitate and trilinolein. Plates were developed in benzene and charred. Five fats and oils gave USM residues that had spots on the TLC plates within the R_f zone for triglycerides: coconut, palm, safflower, and soybean oils, and tallow. These samples were then examined for triglyceride content by the following procedure: 200 μg of USM residue in hexane was applied to a potassium methylate Celite microcolumn to transmethylate glycerices rapidly (6). The effluent was recovered in a syringe and spotted on a Silica Gel G TLC plate, next to a 200 μg spot of the untreated USM. The TLC pattern of both the transesterified and untreated USM were identical, indicating that the spot occupying the triglyceride area on the TLC plate was not a triglyceride.

Diglycerides. Each of the twenty-one USM residues was spotted on Silica Gel G plates in the manner described above. Dipalmitin was spotted next to each residue, and the plates were developed in chloroform. After charring, some of the USM residue samples displayed a spot near that for dipalmitin. These residues were then transesterified (6) and respotted next to a sample of untreated USM. No change could be detected in the TLC patterns from those of the untreated residues.

Monoglycerides. Each USM residue (200 μg) was spotted on Silica Gel G TLC plates which were developed with 5% methanol in dichloromethane. The plates were sprayed with a periodic acid spray to visualize trace amounts of vic-diols (7), and examined under UV light. No spots attributable to monoglycerides were apparent in any of the USM residues.

Soaps. The presence of soap contamination in the USM residues was determined thus: the USM residue was dissolved in dichloromethane and passed over a micro column containing Celite 545 (1 part), phosphoric acid (0.4 part), and water (0.07 part) to regenerate the fatty acids from soaps. The effluent from the column was subjected directly to GLC analysis (6). The GLC trace of the mixture showed no indication of free fatty acids.

Recovery of Two Principal Constituents of USM

Cholesterol. Cholesterol (7.7 mg) was weighed into a mortar containing trioctanoin (4.67 g). Saponification was carried out, and the USM was recovered after elution from a column with dichloromethane. The weight of recovered USM after removal of solvent was 8.7 mg. An equal weight of trioctanoin saponified by itself was found to contain 1 mg USM.

Squalene. Squalene (18 mg) was saponified in the presence of beef tallow (4.56 g) of known USM content. The USM was recovered in the above manner and weighed. The weight of recovered squalene, less that of the beef tallow USM, was 18 mg.

RESULTS AND DISCUSSION

The dry saponification technique was employed on 38 fat and oil samples. The percent unsaponifiable matter (USM) found is shown in Table I. At least two analyses were performed on each sample. Only duplicate values are reported for each oil since triplicate determinations gave essentially the same results. Although the USM content

TABLE II

Results Obtained by the "Dry" and Official Saponification Methods

Oil	Supplier	"Dry" % USM Avg	AOCS Ca6a-40 or Ca6b-53 Avg
Beef oil[a]	A	0.24	0.26[b]
Butterfat	A	0.35	0.35[b]
Rapeseed	B	1.39	1.31[b]
Redfish	C	1.16	0.88[b]
Soybean	B	0.85	0.79[b]
Soybean	D	1.02	0.90[c]
Sperm	A	34.18	35.51[b]
Tall	E	3.11	2.45
Tallow	E	0.58	0.56
Tallow	F	0.72	0.55

[a]See Table I.

[b]Not corrected for the presence of soaps.

[c]Value determined in this laboratory; the other values in this column were supplied by collaborators.

ranged from 0.2-34%, no difficulties in saponification were encountered with this method. Time required for duplicate analyses of typical animal and vegetable oils (ca. 2 hr) was approximately the same regardless of the source of the oil, since elution of the USM from the column is rapid. Saponification time (20 min at 100 C for animal and vegetable oils) may be shorter in ovens with rapid recovery rates; however, this effect was not investigated. Differences were noted in the time required to achieve constant weight of the USM residues. The USM from fish oils, for instance, took longer to reach constant weight than those from tallow or plant oils.

All of the USM residues listed in Table I were analyzed subsequently by several different methods to determine the completeness of the "dry" saponification method and were checked for contamination by soaps. Methods used to detect mono-, di- and triglycerides (Experimental Section) have been shown previously to be sensitive to minute amounts of these substances (5-7), and no trace of glyceride could be detected in any of the USM residues obtained by the dry method. As a precaution against soap carry over, the glass column was first packed with a $CaCl_2$/Celite mixture before the introduction of the samples into the column. Any soap reaching the lower bed was trapped in the $CaCl_2$/Celite matrix. If, however, the $CaCl_2$/Celite bed was omitted, some soap eluted with the USM. For example, the USM from a rapeseed oil sample was found to contain 0.29% oleic acid. Since soap or free fatty acids were not carried over in the USM residue when the $CaCl_2$/Celite bed was used, a titration to correct for these contaminants was not required in the present method.

Studies were also undertaken to determine if two of the principal constituents of the USM fraction of fats and oils could be quantitatively recovered when added to an oil prior to saponification. Cholesterol and squalene were added separately to different oils, the mixture was saponified, and the USM fractions were recovered by the technique described. Both compounds were recovered quantitatively, indicating that this dry saponification technique is not destructive to the major constituents of the USM fraction.

We also attempted to compare the results obtained by the dry technique with those derived from Official AOCS Methods (3,8). Samples of oils which had been evaluated by the suppliers and others for their USM content by AOCS Method Ca6a-40 and/or Ca6b-53 were obtained and analyzed in this laboratory for USM by the dry procedure. One oil also was analyzed in this laboratory by an AOCS method. These results are reported in Table II. Good agreement was found for duplicate assays of all the oils analyzed by the new procedure, but the average values deviate markedly from those obtained by the AOCS Method. Higher average values were generally obtained for the percent USM by our method than by the AOCS technique. Although the comparative data are limited, values obtained by the new procedure are believed to reflect a truer representation of USM content of the fats and oils analyzed, since studies carried out on these residues show that their weights do not reflect the presence of artifacts from the saponification or contamination by soaps.

Application of a modification of the dry column method to determine the amount of USM in commercial fatty acids is under investigation and will be reported subsequently.

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